Pharmacological targeting of the CCL2/CCR2 axis for atheroprotection: a meta-analysis of preclinical studies

Running title: CCL2/CCR2 Inhibition Mitigates Atherosclerosis

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Word count: 7,181 (incl. references).
Number of figures/tables: Five figures, one table.

Abstract

Background: The CC-chemokine ligand-2 (CCL2)/ CC-chemokine receptor-2 (CCR2) axis governs monocyte recruitment to atherosclerotic lesions. Genetic and epidemiological studies show strong associations of CCL2 levels with atherosclerotic disease. Still, experimental studies testing pharmacological inhibition of CCL2 or CCR2 in atheroprone mice apply widely different approaches and report variable results, thus halting clinical translation.

Methods: We systematically searched the literature for studies employing pharmacological CCL2/CCR2 blockade in atheroprone mice and meta-analyzed their effects on lesion size and morphology.

Results: In a meta-analysis of 14 studies testing 11 different agents, CCL2/CCR2 blockade attenuated atherosclerotic lesion size in the aortic root or arch (*g*=-0.75 [-1.17 to -0.32], $p=6\times10^{-4}$; N=171/171 mice in experimental/control group), the carotid (*g*=-2.39 [-4.23 to -0.55], p=0.01; N=24/25), and the femoral artery (*g*=-2.38 [-3.50 to -1.26], $p=3\times10^{-5}$; N=10/10). Furthermore, CCL2/CCR2 inhibition reduced intralesional macrophage accumulation and increased smooth muscle cell content and collagen deposition. The effects of CCL2/CCR2 inhibition on lesion size correlated with reductions in plaque macrophage accumulation, in accord with a prominent role of CCL2/CCR2 signaling in monocyte recruitment. Subgroup analyses showed comparable efficacy of different CCL2- and CCR2-inhibitors in reducing lesion size and intralesional macrophages. The quality assessment revealed high risk of detection bias due to lack of blinding during outcome assessment, as well as evidence of attrition and reporting bias.

Conclusions: Preclinical evidence suggests that pharmacological targeting of CCL2 or CCR2 might lower atherosclerotic lesion burden, but the majority of existing studies suffer major quality issues that highlight the need for additional high-quality research.

Non-standard abbreviations and Acronyms

CCL2 – CC-chemokine ligand 2
CCR2 – CC-chemokine receptor 2
WTD – Western-type diet

Introduction

Stroke and coronary artery disease remain the leading causes of long-term disability and mortality.[1] Multiple lines of experimental and clinical evidence implicate inflammatory mechanisms in atherosclerosis, the predominant pathology underlying cardiovascular disease. Recent clinical trials have provided proof-of-concept for the role of inflammation in atherosclerosis by demonstrating the potential of anti-inflammatory therapies to lower cardiovascular risk.[2, 3] Specifically, canakinumab[2] and colchicine[3, 4] were found to lower the risk of recurrent cardiovascular events in patients with a history of coronary artery disease. While interventional studies in humans have so far mostly focused on the inflammasome-interleukin-1β (IL-1β)-interleukin-6 (IL-6) axis,[5] recent experimental and epidemiological studies place emphasis on other mediators of inflammation, as has specifically been shown for the chemokine system.[6, 7] Targeting alternative inflammatory pathways with a more specific role in atherosclerosis could increase efficacy and improve the safety profile of anti-inflammatory approaches, thus moving them closer to clinical translation.

CC-motif chemokine ligand 2 (CCL2), is one of the first CC family chemokine described and implicated in atherosclerosis.[8, 9] CCL2 primarily acts by binding to CC-chemokine receptor 2 (CCR2) on the surface of classical monocytes, thus mobilizing them from the bone marrow to the circulation and attracting them to sites of inflammation[10] including the arterial subendothelium.[8, 11] CCL2/CCR2 signaling governs rolling and adhesion of monocytes on the endothelial lining of atherosclerotic lesions.[12] Hyperlipidemic atheroprone mice deficient for either *Ccl2*[13] or *Ccr2*[9] exhibit substantial reductions in the number and size of atherosclerotic lesions, as well as reductions in lipid deposition and macrophage accumulation in the arterial walls, thus supporting a causal role of the CCL2/CCR2 pathway in atherogenesis.

The potential importance of these findings for the development of therapeutic strategies is illustrated by recent studies demonstrating a causal role of CCL2 in human atherosclerosis. First, using a Mendelian randomization approach, we recently found higher genetically proxied circulating levels of CCL2 to be associated with a higher risk of ischemic stroke, in particular large artery stroke, and a higher risk of coronary artery disease and myocardial infarction.[14] Second, higher measured levels of circulating CCL2 were associated with a higher risk of ischemic stroke, coronary artery disease, and cardiovascular mortality in population-based cohorts of individuals free of cardiovascular disease at baseline.[15, 16] Third, CCL2 levels quantified in atherosclerotic plaques from individuals undergoing carotid endarterectomy showed significant associations with histopathological, clinical, and molecular features of plaque vulnerability.[17]

While these studies identify the CCL2/CCR2 axis as a promising pharmacological target for the treatment of atherosclerosis, there are only limited data from randomized trials specifically targeting this pathway in the context of human atherosclerosis. In a study on 108 patients with cardiovascular risk factors and high circulating levels of high-sensitivity C-reactive protein (hsCRP), those treated with a single intravenous infusion of MLN1202, a humanized monoclonal antibody against CCR2, exhibited significant reductions in hsCRP levels after four weeks and continuing through 12 weeks after dosing.[18] However, this phase II trial was not designed to investigate clinical endpoints.

Preclinical studies have explored various pharmacological approaches targeting the CCL2/CCR2 axis in models of atherosclerosis. Still, these studies reported largely variable and partly inconsistent results, possibly reflecting differences in the properties of the individual pharmacological agents, the selected drug targets (CCL2 or CCR2), the molecular sites in their structures targeted by the agents, the animal models under study, lesion stages at initiation of the intervention, duration of treatment, and the vascular beds under examination. Against this background, we aimed to analyze the available evidence from preclinical studies testing pharmacological inhibition of the CCL2/CCR2 pathway in atherosclerosis-prone mice and quantify the effects of the interventions on lesion size and cellular and extracellular plaque components (macrophage accumulation, smooth muscle cell content, and collagen

deposition). We further aimed to detect potential sources of heterogeneity of their efficacy including those related to pharmacological properties of the inhibitor, vascular bed, animal model, duration of treatment, and stage of atherosclerosis. We therefore performed a systematic review and meta-analysis of preclinical studies in an effort to inform the design of future clinical trials in humans.

Methods

This meta-analysis follows the PRISMA guidelines on systematic reviews (**Online Table I**). The dataset underlying this work is available online as an online dataset.

Search strategy

The SyRF 9-step outline and SYRCLE's protocol template for conception of animal meta-analyses[19] were used to design the search strategy, study and outcome selection, and statistical processing of the extracted data for this systematic review and meta-analysis. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)[20] were used as reporting guidelines of this meta-analysis (**Online Table I**). To identify eligible articles, we screened MEDLINE and EMBASE from their inception to January 20th, 2022 without restrictions in language or publication year, using the following predefined search strategy: ("CC chemokine ligand 2" OR "C-C chemokine ligand 2" OR "C-C motif chemokine ligand 2" OR "C-C motif chemokine receptor type 2" OR "C-C chemokine receptor type 2" OR "CC chemokine receptor type 2" OR "monocyte chemoattractant protein" OR "monocyte chemotactic protein" OR CCL2 OR MCP-1 OR MCP1 OR CCR2) AND (atherogenesis OR atherosclerosis OR atheroprogression OR atherosclerotic OR plaque OR stroke OR ((cardiovascular OR ischemic OR cerebrovascular OR coronary) AND disease) OR (myocardial AND infarction)). A published search filter was employed to limit displayed entries to those referring to animal experiments.[21] Additionally, the reference lists of all eligible studies were screened. Eligible articles were evaluated for potential overlap of data. One reviewer (L.Z.) performed the initial screening and all potentially eligible articles were further independently screened by an additional reviewer (M.G.); differences were resolved in consensus.

Eligibility criteria

The eligibility criteria applied for our study selection strategy were pre-defined before the start of the literature search.

Population

Articles were deemed eligible if they described experimental inhibition of CCL2 or CCR2 in an *in vivo* mouse model of atherosclerosis. Specifically, eligible studies were required to use atherosclerosis-prone mouse models, such as *Apoe*$^{-/-}$, *Ldlr*$^{-/-}$, or ApoE3Leiden mice that were fed a normal laboratory diet or high-fat "Western-type" diet (WTD). Models of accelerated atherosclerosis following arterial injury in atherosclerosis-prone mice were also considered eligible.[22, 23] Studies referring to other animals beyond mice were not included in this review.

Intervention

Eligible studies had to explore the effects of a pharmacological intervention directly interfering with and inhibiting CCL2 or CCR2, such as orthosteric or allosteric receptor antagonists, competitive inhibitors of chemokine-receptor interaction, or antibodies. Studies that made use of gene therapy by means of transfecting plasmids, such as 7ND,[24-27] were also considered eligible, provided that the encoded protein was a direct inhibitor of CCL2 or CCR2. Studies that examined pharmacological agents or nutritional compounds that indirectly downregulate the CCL2/CCR2 axis by interfering with upstream agents or downregulated CCL2 or CCR2 expression were deemed ineligible. Eligible studies also required a control group of animals which were injected with a vehicle or were fed an inhibitor-free diet.

Outcomes

Eligible studies needed to provide a quantified measurement of atherosclerotic plaque burden as an outcome. Histopathological quantification of lesion size/area, plaque size/area, neointimal area, or lipid-staining area following hematoxylin-eosin, Oil Red O, trichrome or pentachrome staining of vessel cross-sections were required for inclusion in the meta-analysis. Studies providing measurements of intima/media ratio were excluded, because these readouts fail to distinguish atherosclerotic plaque burden from intimal hyperplasia and vascular thickening. Apart from the well-established lesion quantification in the aortic root or arch, studies measuring carotid or femoral artery lesions were also included in the meta-analysis.

Additional predefined outcomes entailed plaque cellular and extracellular components including macrophage accumulation (expressed as Mac2/3-positive or Moma-2/3-positive content), smooth muscle cell content (expressed as smooth-muscle-actin-positive content), and collagen deposition. Plaque feature outcomes were only included if they were normalized to plaque size. We further explored the following as secondary outcomes: effects of the intervention on additional measurements that had not been predetermined, when explored by at least three individual studies. These included body weight, plasma cholesterol and triglyceride levels, circulating monocyte count, plasma CCL2 levels, and aortic expression of CCR2, IL-6, and TNF-α.

Study quality assessment

We examined potential sources of bias with the SYRCLE risk of bias tool that was specifically designed for preclinical studies.[28] The tool evaluates studies for selection bias (3 items), performance bias (2 items), detection bias (2 items), attrition bias (1 item), reporting bias (1 item), and other sources of bias (1 item). Full texts, figure legends and supplementary materials were considered in the risk of bias assessment. The specific criteria used in risk of bias assessment are shown in **Online Table II**. Furthermore, we explored whether the included studies followed the ARRIVE guidelines for reporting animal research for sample size, randomization, and blinding.

Data abstraction

Absolute values, number of specimens, and either standard error or standard deviation in both intervention and control groups were extracted for each outcome (**Online Table III**). Where numerical data was not available, values were extracted from figures. Additionally, information pertaining to experimental setup such as inhibitor used and its target (CCL2 or CCR2), blood vessel under examination, mouse background and genetic model, sex, type of diet and (where applicable) start and duration of WTD feeding, start and duration of inhibitor administration, and additional pharmacological interventions were recorded for each experimental group. Where abstractable data were not presented in the published article or the supplementary materials, the corresponding author was contacted for providing the required information. One reviewer (L.Z.) performed the data abstraction and all data were further checked by a second reviewer (M.G.).

Meta-analysis

For all studies and outcomes, we calculated standardized mean differences (Hedges' *g*) between the experimental and the control groups using the Hedges approach to account for the small sample sizes. We then pooled the individual study estimates using DerSimonian-Laird random effects models to account for the expected heterogeneity between studies. For the main outcomes (lesion size and plaque characteristics), we performed the analysis separately for the different examined vessels (aortic root or arch, carotid artery, femoral artery). We calculated between-study heterogeneity with the I^2 and the Cochran *Q* statistic. I^2 exceeding 50% or 75% was considered as moderate and high heterogeneity, respectively.[29] Finally, we performed Egger regression to explore potential small-study effects in our main analysis that would indicate presence of publication bias.[30] Funnel plots were also created and visually inspected for asymmetry due to small-study effects.

To account for potential sources of heterogeneity, we performed a series of subgroup and meta-regression analyses. Specifically, we explored if the stage of lesion progression at the time of intervention start influenced the intervention effects. Lesion stage was classified as early, intermediate or advanced on the basis of mouse model, diet used, and age at intervention: *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice fed a normal laboratory diet were assumed to exhibit early lesions until they were 15 weeks old, intermediate lesions between 15-20 weeks, and advanced lesions after 20 weeks of age. The respective intervals in WTD-fed mice were until 10 weeks (early lesions); 10-15 weeks (intermediate lesions), and after 15 weeks (advanced lesions).[31, 32] We further performed subgroup analyses by target of intervention (CCL2 vs. CCR2), animal model (*Apoe*$^{-/-}$ vs. *Ldlr*$^{-/-}$), type of diet (normal laboratory vs. WTD) and sex. Finally, we carried out meta-regression analyses, exploring whether the effects of the interventions on plaque characteristics or other secondary outcomes, could explain heterogeneity in the effects on lesion size.

All analyses were performed using Stata 16.1 (College Station, United States).

Data availability

The dataset generated from the data abstraction of the included studies is available as a separate Online Supplementary File. The Stata code used for our analyses is available on Dataverse: https://doi.org/10.7910/DVN/KMKD0J.

Results

The results of the search strategy are summarized in **Fig. 1**. The PubMed search returned 4,551 entries, out of which 290 articles were assessed for eligibility through inspection of their full texts. Of them, 16 articles met our eligibility criteria. Two of them presented data already available in another publication,[33, 34] whereas one article[35] did not present any abstractable data. One additional article[36] was identified through screening the reference lists of the eligible studies. A total of 14 articles[11, 24-27, 36-44] were eventually deemed eligible for inclusion in our systematic review and meta-analysis.

Characteristics of eligible studies

Table 1 summarizes key characteristics of the included studies, which were published between 2001 and 2018. All 14 eligible studies used hyperlipidemic mouse models of atherosclerosis. Specifically, 12 studies used *Apoe*$^{-/-}$ mice, one *Ldlr*$^{-/-}$ mice, and one ApoE3Leiden mice. Nine studies relied on WTD feeding in their experimental setup, whereas in four studies mice were fed normal laboratory diet, with one study not specifying any type of diet. Most of the studies that used a WTD initiated high-fat feeding before the age of ten weeks. Timing of intervention and duration of treatment differed widely in the eligible studies ranging from four to 30 weeks (age at initiation of treatment) and three to 12 weeks (treatment duration), respectively.

Twelve studies targeted CCR2 with an inhibitory compound. Three studies used commercially available inhibitors.[36, 43, 44] Specifically, Yamashita et al.[44] tested Propagermanium, an organometallic, which has been used in the treatment of chronic hepatitis B,[45] whereas Van Wanrooij et al. used TAK-779, a small molecule inhibitor of CCR2, CCR5, and CXCR3[38] that is under examination as an HIV entry inhibitor.[36] Winter et al.[43] tested RS102895, a CCR2 small molecule antagonist in a chronopharmacological study. Four studies examined proprietary inhibitors that were developed in-house[37, 38, 41, 42] and four studies utilized a plasmid encoding for an N-terminal deletion mutant of CCL2 (termed 7ND) as a therapeutic compound,[24-27] which inhibits CCL2 signaling by functioning as a dominant-negative inhibitor of CCL2.[46, 47] Finally, Liehn et al.[40] opted for a similar approach using a recombinant N-terminal truncate of CCL2. Only two studies used approaches directly targeting CCL2: Lutgens et al.[11] employed a monoclonal anti-CCL2 antibody, whereas Cynis et al.[39] used a small molecule blocking essential posttranslational modifications on the N-terminus of CCL2. Regarding study outcomes, all but two studies examined plaque burden either in the aortic root or arch, whereas three studies explored lesions in the carotid artery. Cynis et al.[39] opted for a femoral artery wire injury model in ApoE3Leiden mice, where lesion development was artificially induced and accelerated. Similarly, Liehn et al.[40] conducted wire injury in the carotid artery of *Apoe*$^{-/-}$ mice.

Inhibiting CCL2 or CCR2 reduces lesion size and skews plaques towards a stable phenotype

Twenty-two treatment arms from all 14 studies were included in the meta-analysis for lesion size, as displayed in **Fig. 2**. Blockade of CCL2 or CCR2 resulted in a significant decrease in atherosclerotic lesion size in the aortic root or arch (g=-0.75 [-1.17 to -0.32], $p=6\times10^{-4}$), as derived after pooling 18 study arms (171 animals in experimental group, 171 controls). Significant decreases were also found in both the carotid (g=-2.39 [-4.23 to -0.55], p=0.01, k= 3 study arms, 24 animals in experimental group, 25 controls) and femoral arteries (g=-2.38 [-3.50 to -1.26], $p=3\times10^{-5}$, k= 1 study arm, 10 animals in experimental group, 10 controls). There was a significant difference in the effects of CCL2/CCR2 inhibition across the three vascular beds (p=0.01) with larger effects seen in the carotid and femoral arteries, as compared to the aortic root and arch.

CCL2/CCR2 inhibition further reduced the intralesional macrophage accumulation in the aortic arch and root (g=-0.76 [-1.11 to -0.41], $p=2\times10^{-5}$, k= 12 study arms, 112 animals in experimental group, 111 controls) (**Fig. 3**), while leading to an increase in collagen deposition (g=0.70 [0.16 to 1.24], p=0.011, k= 6 study arms, 60 animals in experimental group, 60

controls) and smooth-muscle cell content (g=0.95 [0.24 to 1.66], p=0.009, k= 6 study arms, 61 animals in experimental group, 61 controls), consistent with a more stable plaque phenotype.[48]

Associations between CCL2/CCR2 inhibition and secondary outcomes are presented in **Online Figure I** and **Online Table III**. The experimental groups did not undergo changes in body weight, plasma triglycerides or blood monocytes. However, there was a significant increase in CCL2 plasma levels across studies inhibiting CCR2 and a significant decrease in IL-6 expression levels within plaques. There was a borderline association between CCL2/CCR2 inhibition and lower plasma total cholesterol levels.

Subgroup analyses reveal no differences by lesion stage or intervention target

There was at least moderate heterogeneity (I^2>50%) for all main outcomes except for macrophage accumulation (I^2=42%). To explore whether other study variables could explain the between-study differences we performed a series of subgroup analyses (**Fig. 4** and **Online Table IV**). There were no significant differences between subgroups of different stages of atherosclerosis progression (early, intermediate, advanced) at the time of onset of intervention, although there was a tendency for smaller effect sizes in mice with more advanced lesions. Similarly, we observed no difference in the effects of intervention on lesion size in the aortic arch or root between targets of intervention, with both CCL2 and CCR2 inhibition showing significant reductions. Lesion size reduction differed significantly between WTD-fed mice and mice fed normal laboratory diet (p=0.048) with the latter showing no significant reduction in lesion size. All but one study examining aortic lesions used *Apoe*$^{-/-}$ models of atherosclerosis, but the single study using *Ldlr*$^{-/-}$ mice also showed a significant reduction in lesion size. No significant differences in effects were detected between male- and female-specific analyses. None of the subgroup analyses resolved the heterogeneity between studies. A meta-regression analysis revealed an association between longer intervention duration and larger atheroprotective effects on lesion size (β=-0.153 [-0.285 to -0.021], p=0.023; **Online Figure IIA**), but failed to account for study heterogeneity (residual I^2=67%).

Effects of CCL2/CCR2 inhibition on intralesional macrophage accumulation predict the reductions in lesion size

To further explore sources of the derived heterogeneity, we performed meta-regression analyses exploring the associations between the effects of CCL2/CCR2 inhibition on plaque features and their effect on lesion size. We found a significant association between the effects of different interventions on macrophage accumulation within plaques and the effects on the overall aortic lesion size (β=0.789 [0.263 to 1.314], p=0.003; **Fig. 5**). Residual heterogeneity (I^2) after meta-regression was 27% compared to the initial 73%, thus invoking the notion that differences across the interventions in their effects on macrophage accumulation within plaques could explain 62% of the differences in overall effect on lesion size. There was no significant association between the effects of CCL2/CCR2 inhibition on plasma CCL2 levels and its effect on lesion size (**Online Figure IIB**).

Publication bias and risk of bias assessment

Applying the Egger's test, we detected a significant small-study effect (β=-7.95 [-12.08 to -3.82] p=0.0002) in the main analysis exploring the effects of CCL2/CCR2 inhibition on aortic lesion size, thus indicating presence of potential publication bias. After visual inspection of the respective funnel plot (**Online Figure III**), we explored whether a single outlier study[44] could account for the observed effect. Following exclusion of this study, the observed small-study effect was attenuated (β=-5.79, [-11.77 to 0.19], p=0.058), while the overall effect of CCL2/CCR2 inhibition on aortic lesion size remained stable (g=-0.55, [0.93 to -0.17], p=0.005).

Finally, all eligible studies underwent a thorough quality assessment with the SYRCLE risk of bias tool, which was used to define the assessment criteria we lay out in **Online Table II**.[28] The detailed results are presented in **Online Figure IV**. Importantly, there was evidence of

high risk of detection bias caused by a lack of assessor blinding during outcome assessment in eleven eligible studies. Furthermore, we detected high risk of attrition bias in eight studies, which failed to report exact sample sizes for every experiment or reasons for differing sample sizes across experiments of the same cohorts. All eligible studies were also assigned a high risk of reporting bias, because of the lack of a published pre-defined study protocol. The tool items referring to selection or performance bias, as well as another aspect of detection bias represented by randomness of outcome assessment order, could not be assessed for most eligible studies due to insufficient information provided within the respective publications. The adherence of the included studies to the ARRIVE guidelines for sample size, randomization, and blinding is summarized in **Online Figure V**. While all of the studies reported on samples sizes across tested subgroups, no study reported *a priori* sample size calculations. Furthermore, only 3 of the 14 studies clearly reported their randomization and blinding protocols.

Discussion

Pooling data from 14 preclinical studies of experimental atherosclerosis, we found that pharmacological blockade of CCL2 or CCR2 in mice leads to a significant reduction in atherosclerotic lesions in the aorta, the carotid, and the femoral artery. Furthermore, pharmacological inhibition of CCL2 or CCR2 is associated with reductions in intralesional macrophage accumulation and increases in plaque smooth muscle cell content and collagen deposition. These effects were similar when targeting either CCL2 or CCR2 but were stronger for lesions in the carotid and femoral arteries than in the aorta. While there was substantial heterogeneity in the extent of CCL2/CCR2 inhibition on atherosclerotic lesion size, these effects were highly correlated with the effects of the interventions on macrophage accumulation within plaques, thus supporting the notion that intralesional macrophage reduction can serve as a surrogate marker of efficacy of CCL2/CCR2 inhibition. Still, despite the promising results, our quality assessment detected sources of detection, attrition, and reporting bias in the majority of existing studies.

The consistently large effects of pharmacological CCL2/CCR2 inhibition on atherosclerotic lesions across studies with different designs and across different vascular beds, when seen in conjunction with previous findings in *Ccl2*$^{-/-}$[13] or *Ccr2*$^{-/-}$[9] mice testing the genetic deletion of the ligand or the receptor, provide strong preclinical support for the candidacy of CCL2/CCR2 signaling as a promising target in atherosclerosis. While data from clinical trials remain limited,[18] recent results from genetic[14] and epidemiological[15, 16] studies emphasize a causal role of the CCL2/CCR2 axis in human atherosclerosis. Hence, there is coherent evidence from preclinical, genetic, epidemiological, and early-phase clinical trials that targeting the CCL2/CCR2 pathway may be a viable strategy to mitigate the risk of atherosclerotic disease.

Interestingly, we found stronger attenuating effects of CCL2/CCR2 inhibition on atherosclerotic lesions in the carotid artery, as compared to lesions in the aortic arch and root. While these findings cannot be directly translated to humans, they are consistent with the stronger associations we previously found between both measured and genetically determined CCL2 circulating levels and risk of ischemic stroke, as compared to coronary artery disease and myocardial infarction.[14-16] Despite the common mechanisms underlying atherogenesis and atheroprogression across different vascular territories, differences in the effects of established risk factors, such as smoking and hypertension, on atherosclerotic manifestations from different vascular beds are well-known.[50] Whether pharmacological targeting of the CCL2/CCR2 axis and inflammation in humans differentially affects atherosclerotic lesion formation in different vessels would need to be further explored in future studies. Still, this could have implications for the selection of the right population for future clinical trials.

Aside from its influence on lesions size, CCL2/CCR2 inhibition further exerted an effect on plaque composition. Specifically, mice in the intervention arms exhibited a lower macrophage accumulation and a higher smooth muscle cell and collagen content, consistent with a smaller core and a thicker fibrous cap, both characteristics of a plaque less vulnerable to rupture and subsequent complications like acute ischemic stroke or coronary syndromes.[48] Our results are consistent with those of a recent cross-sectional study of plaque samples from patients undergoing carotid endarterectomy, which showed associations between CCL2 levels within plaques and histopathological features of plaque vulnerability.[17] Thus, these data support a role of CCL2/CCR2 beyond the early stages of atherogenesis and highlight the potential benefits of targeting this axis even in patients with established atherosclerotic disease in future trials.

In a meta-regression analysis, we found the heterogeneity of the effects of CCL2/CCR2 inhibition on aortic lesion size to be to a large extent explained by the effects on plaque macrophage accumulation. This observation agrees with the key role of the CCL2/CCR2 axis in attracting monocytes to the atherosclerotic lesion, but also indicates that the effects of pharmacological approaches targeting the CCL2/CCR2 axis on intralesional macrophage

accumulation could be used as a surrogate marker of the overall efficacy of CCL2/CCR2 inhibition. The latter could have implications for the design of future early-phase clinical trials and the identification of a proper readout for drug response and efficacy beyond clinical endpoints that would require very large sample sizes.

Another important finding of the current meta-analysis is the lack of heterogeneity in efficacy between studies targeting either CCL2 or CCR2. The consistency in the effects of molecules targeting either the ligand CCL2 or its receptor CCR2 for either decreasing lesion size or improving the plaque composition indicate no superiority of one approach over the other in animal models of atherosclerosis. This is important given the different structural and targeting properties of the pharmaceutical agents employed. Features such as surface coverage, binding affinity, or bioavailability differ substantially between antibodies, orthosteric small molecule inhibitors, or decoy ligands. Also, it should be noted that there are fewer agents targeting chemokine ligands, as compared to chemokine receptors,[51] which is also reflected in the low number of studies inhibiting CCL2 in our meta-analysis. Moving towards clinical trials in humans, it would be important to consider both approaches as potentially promising.

Our study has limitations. First, there was considerable between-study heterogeneity in almost all analyzed outcomes, which could bias the derived effect estimates. It is possible that differences in experimental design as well as in the efficacy of the tested interventions underly this heterogeneity. More specifically, there were differences in the background strains of mice, models that were examined, the stages of lesion progression at the time of intervention, the duration of treatment, the pharmacological properties of the different CCL2/CCR2 inhibitors, the mode of administration of the different agents, the dosage, the vessels that were examined, and even the methods for quantifying lesion formation. While we aimed to account for these differences in subgroup and meta-regression analyses, this was not possible for all of these factors, whereas several study subgroups were too small for meaningful analyses. For instance, the finding that there were no significant differences in lesion size reduction between early, intermediate, and advanced lesions may have been driven by the small number of studies reporting effects on already established atherosclerotic plaques. While our subgroup analyses based on the time of intervention was rather underpowered, individual studies support a larger impact when treatment is started at early stages before lesions have been formed, corroborating the important role of the CCL2/CCR2 axis in atherosclerotic lesion initiation.[37, 49] There were also substantial differences in fat source, percentage, and cholesterol content of the atherogenic Western-type diets that were used by the included studies, which may explain part of the observed heterogeneity. The small number of studies with detailed information on the exact composition of the Western-type diet prohibited a meaningful analysis of the impact of diet composition on our results. Second, there was evidence of small-study effects indicating publication bias. While publication bias could indeed influence the effect estimates, we found the small-study effect to be primarily driven by a single outlier study. Reassuringly, the effects were only slightly attenuated after exclusion of this study from the meta-analysis. Third, some of our analyses, such as the analyses of lesions in the carotid and femoral arteries, the analyses for CCL2 inhibition, the subgroup analyses per stage of atherosclerotic lesions, and some meta-regression analyses were based on a rather small number of study arms and are thus limited by low statistical power. The disproportional number of studies exploring lesions in the aortic root or arch is to be expected because of the higher prevalence of atherosclerotic lesions in the aorta of genetically atheroprone mice, as compared to other vessels, which makes it the vascular bed of choice for the majority of studies exploring mouse models of atherosclerosis.[52] Fourth, the lesion staging used in the subgroup analysis relied on the age of mice, feeding, and treatment durations rather than histopathological lesion assessment due to paucity of data. Fifth, the exclusion of studies not meeting our eligibility criteria might have biased our results. However, because our selection strategy was pre-defined before the start of the literature search and our criteria were relatively broad, as reflected in the considerable between-study differences in experimental design, we believe that there is no systematic bias towards a selective inclusion of published studies with specific findings. Lastly, agents used in some studies, like 7ND, appear impractical for therapeutic approaches in humans compared to small molecule inhibitors.

An important element of our review that should be highlighted is the high risk of several forms of bias in the included studies, as detected in our quality assessment. We found the majority of the included studies to fulfill few of the quality criteria and to be vulnerable to detection, attrition, and reporting bias. For example, none of the included studies was performed according to a pre-published protocol, whereas the eligible studies provided no information on random animal housing, caretaker blinding, and random outcome assessment. This necessitates cautious interpretation of the findings, as sources of bias in preclinical studies can contribute to lack of translation of promising preclinical experiments into successful clinical trials.[53]

In view of these limitations, our findings offer key insights into the impact of CCL2/CCR2 inhibition on atheroprotection beyond the results of individual studies entering the meta-analysis. Specifically, these novel findings may have important implications for the transition of strategies targeting the CCL2/CCR2 axis for atheroprotection towards clinical testing. First, the lack of a difference in the efficiency of agents targeting either CCL2 or CCR2 highlights the importance of further investment in the development of agents targeting either molecules and implies that efficient agents against either target could be promoted for clinical testing. Second, our finding that the efficiency of different agents for reducing plaque size is proportional to their effects on macrophage accumulation opens a new pathway for development of biomarkers capturing plaque macrophage content, which could be used in trials testing such agents. Imaging methods currently under development, such as PET-based assessment of plaque inflammation could prove useful in this regard.[54] Third, pooling studies in a meta-analysis offered sufficient power to detect highly consistent effects for an effect of CCL2/CCR2 inhibition on plaque features beyond plaque size, which emphasizes the potential utility of this approach for patients with established atherosclerotic lesions. Fourth, our finding of a stronger effect on carotid and femoral lesions has implications for the selection of target populations that could benefited from such approaches. Beyond patients with coronary artery disease that have been the primary target population of previous anti-inflammatory trials for atheroprotection,[2-4, 55] our results suggest that clinical testing could likewise be expanded to other populations, such as patients with carotid stenosis, large artery atherosclerotic stroke, and established peripheral artery disease.

In conclusion, preclinical evidence supports a potential atheroprotective effect of the pharmacological targeting of the CCL2/CCR2 axis in mouse models of atherosclerosis. However, our systematic review detected high risk of bias in the published studies, thus highlighting the need for additional research based on robust methodology in this translationally relevant field. While still weak, this preclinical evidence adds to the recent data from human studies that imply a translational potential of targeting CCL2/CCR2 signaling in atherosclerotic disease and provides insights for informing the design of future clinical trials.

Acknowledgements

None.

Sources of funding

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) through grants for the Collaborative Research Center 1123 (CRC1123, fellowship IRTG1123 to L.Z., project B03 to Y.A., project A03 to J.B., project B03 to M.D.). L.Z. received a graduate scholarship from the Medical Faculty of the Ludwig-Maximilians-University (LMU) Munich. Y.A. was supported by grants from the FöFoLe program of LMU Munich (FöFoLe 921) and the Friedrich Baur-Stiftung. MG acknowledges support in form of a Walter-Benjamin fellowship from DFG (GZ: GE 3461/1-1) and from the FöFoLe program of LMU Munich (Reg.-Nr. 1120).

Disclosures

None.

Supplemental Materials

Online Tables I-IV
Online Figures I-IV
Supplemental References
Major Resources Table
Meta-analysis dataset

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Highlights

- Pharmacological inhibition of either CCL2 or CCR2 is associated with reduced lesion sizes in hyperlipidemic mouse models of atherosclerosis.
- CCL2/CCR2 inhibition further changes plaque features like macrophage accumulation, collagen deposition, and smooth-muscle cell content.
- Effects on CCL2/CCR2 inhibition on macrophage accumulation are significantly associated with effects on lesion size.
- There was no sufficient power to detect differences in the effects of CCL2 or CCR2 inhibition by stage of atherosclerosis at the time of treatment onset.
- The majority of existing studies suffer major quality issues that highlight the need for additional high-quality research.

Table 1. **Descriptive characteristics of eligible studies included in the meta-analysis.** N/A = Information not provided in the study; m = male, f = female; WTD = Western-type diet; d = day; p.o. = oral gavage, i.p. = peritoneal injection, i.m. = intramuscular injection, s.c. = subcutaneous injection.

First author, year	Mouse strain & model	Sex	Diet (manufacturer & for WTD fat composition, where available)	Start of diet/ weeks	Intervention	Dosage, route, interval	Start of intervention/weeks (duration/ weeks)	Study groups (n)	Lesion site(s)
AIELLO, 2010[37]	C57BL/6, *Apoe*$^{-/-}$	m	normal laboratory diet (Purina Prolab® RMH 3000)		INCB-3344 (small molecule CCR2 antagonist)	50mg kg^{-1} d^{-1}, p.o.	7 (4) 10 (6) 10 (10) 20 (6)	4-week treatment (8) & control (8) 10-week-old, 6-week treatment (7) & control (11) 20-week-old, 6-week treatment (8) & control (10) 10-week treatment (7) & control (8)	Aortic root, brachiocephalic artery (20-week-old mice, 6-week treatment)
BOT, 2017[38]	N/A *Apoe*$^{-/-}$	m	WTD (SDS, 15% cocoa butter, 0.25% cholesterol)	10-12	15a (small molecule CCR2 antagonist)	5mg kg^{-1}, i.p. 1x daily	10-12 (4)	Treatment (9) & control (10)	Aortic root, Carotid artery (cuff placement)
CYNIS, 2011[39]	C57BL/6J, ApoE3 Leiden	m	WTD (manufacturer N/A, 15% cocoa butter, 1% corn oil, 1% cholesterol, 2% choline, 0.05% cholate)	12	PQ50 (small molecule glutaminyl cyclase/iso-glutaminyl cyclase inhibitor)	2,4mg mL^{-1} in drinking water, p.o. for 7 days, then 1,2mg mL^{-1}	12 (5)	Treatment (10) & control (10)	Femoral artery (cuff placement)
INOUE, 2002[27]	C57BL/6, *Apoe*$^{-/-}$	N/A	normal laboratory diet (Oriental Yeast)		7ND (plasmid encoding an N-terminal CCL2 deletion variant)	100µg, i.m. 1x biweekly with electroporation	20 (8)	Treatment (10) & control (10)	Aortic arch
LIEHN, 2010[40]	N/A, *Apoe*$^{-/-}$	N/A	WTD (manufacturer and composition not specified)		PA508 (recombinant CCL2 variant (CCL2-based "decoy" chemokine incapable of CCR2 activation)	10µg, i.p. 1x daily	8 (3)	Treatment (5) & control (5)	Carotid artery (wire injury)
LUTGENS, 2005[11]	C57BL/6 *Apoe*$^{-/-}$	m	N/A		11K2 (monoclonal CCL2 antibody)	100µg, i.p. 2x/week	5 (12) 17 (12)	5-week-old, early treatment & control 17-week-old, delayed treatment (all 15 per group)	Aortic arch

Ni, 2001[24]	C57BL/6J, *Apoe*$^{-/-}$	N/A	WTD (Oriental Yeast, 20% fat, 0.15% cholesterol)	7-8	7ND (plasmid encoding an N-terminal CCL2 deletion mutant; encapsulated in 7HVJ liposome)	5µg, i.m. 1x every 3 weeks	7-8 (6)	Treatment (8) & control (8)	Aortic root
Ni, 2004[25]	C57BL/6 *Apoe*$^{-/-}$	m	normal laboratory diet		7ND (plasmid encoding an N-terminal CCL2 deletion mutant)	100µg, i.m. 1x biweekly with electroporation	30 (4)	Saline infusion, treatment & control Angiotensin-II infusion, treatment & control (all 10)	Aortic root
Okamoto, 2012[41]	C57BLKS/J, *Apoe*$^{-/-}$	m	WTD (Oriental Yeast, 15% fat, 1.25% cholesterol)	4	TLK19705 (small molecule CCR2 antagonist)	10mg kg^{-1} d^{-1}, p.o.	4 (8)	Treatment (10) & control (8)	Aortic root
Olzinski, 2010[42]	N/A, *Apoe*$^{-/-}$, human CCR2 knock-in	N/A	WTD (21% milk fat, 0.2% cholesterol)	22-24	GSK1344386B (small molecule CCR2 antagonist)	10mg kg^{-1} d^{-1}, p.o.	22-24 (5)	Treatment (20) & control (20), both Angiotensin-II-treated	Aortic root
de Waard, 2010[26]	N/A, *Apoe*$^{-/-}$	m	WTD (SDS, composition N/A)	8-10	7ND (plasmid encoding an N-terminal CCL2 deletion mutant)	unknown, i.m. once	12-14 (4-5)	Treatment & control, both Angiotensin-II-treated (26 total)	Aortic root & arch
van Wanrooij, 2005[36]	N/A, *Ldlr*$^{-/-}$	f	WTD (manufacturer N/A 15% cocoa butter, 0.25% cholesterol)	15	TAK-779 (small molecule CCR2/CCR5/ CXCR3 antagonist)	100µg, s.c. every 2 days	17 (6) 15 (6)	15-week-old treatment & control, 17-week-old, collar-implanted treatment & control (all 10)	Aortic root, carotid artery (cuff placement)
Winter, 2018[43]	C57BL/6J, *Apoe*$^{-/-}$	m&f	WTD (ssniff cat. # E15721, 21% fat, 0.15% cholesterol)	8	RS102895 (small molecule CCR2 antagonist)	5mg kg^{-1}, i.p. 1x daily	8 (4)	Zeitgeber time 5 & 17 both treatment & control each (all 8)	Aortic root
Yamashita, 2002[44]	C57BL/6, *Apoe*$^{-/-}$	N/A	WTD (Oriental Yeast, normal laboratory diet plus 7.5% cocoa butter, 1.25% cholesterol, 0.5% cholate)	4	Propagermanium (small molecule CCR2 antagonist)	0,005% of diet, p.o.	4 (8) 4 (12)	8-week & 12-week both treatment & control each (all 8)	Aortic root

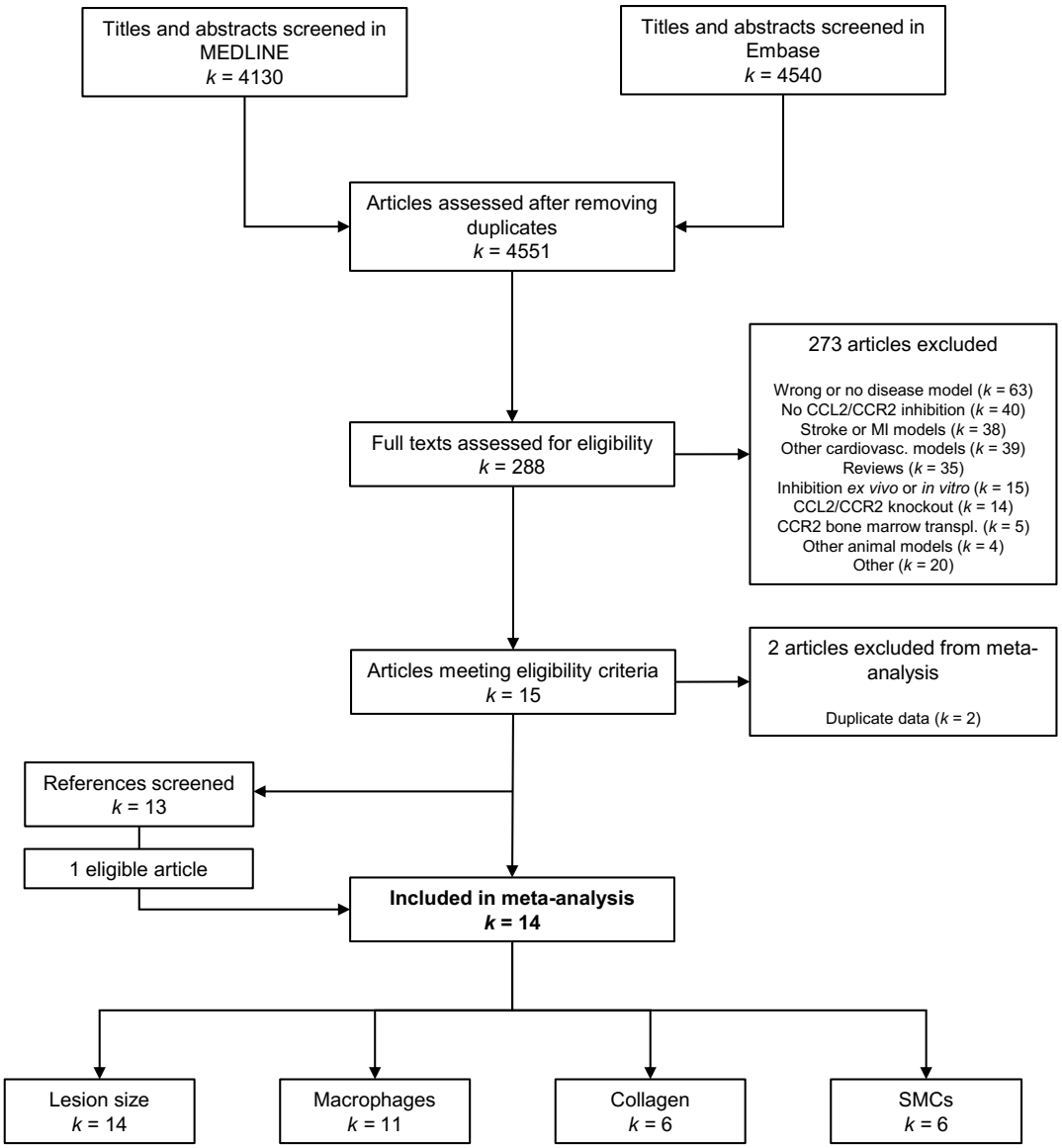

Figure 1. Flowchart of the study selection process. The search was performed in MEDLINE through the PubMed engine as well as Embase. Articles were evaluated for eligibility on the basis of their titles, abstracts, and full texts. k = number of articles. SMCs = smooth-muscle cells.

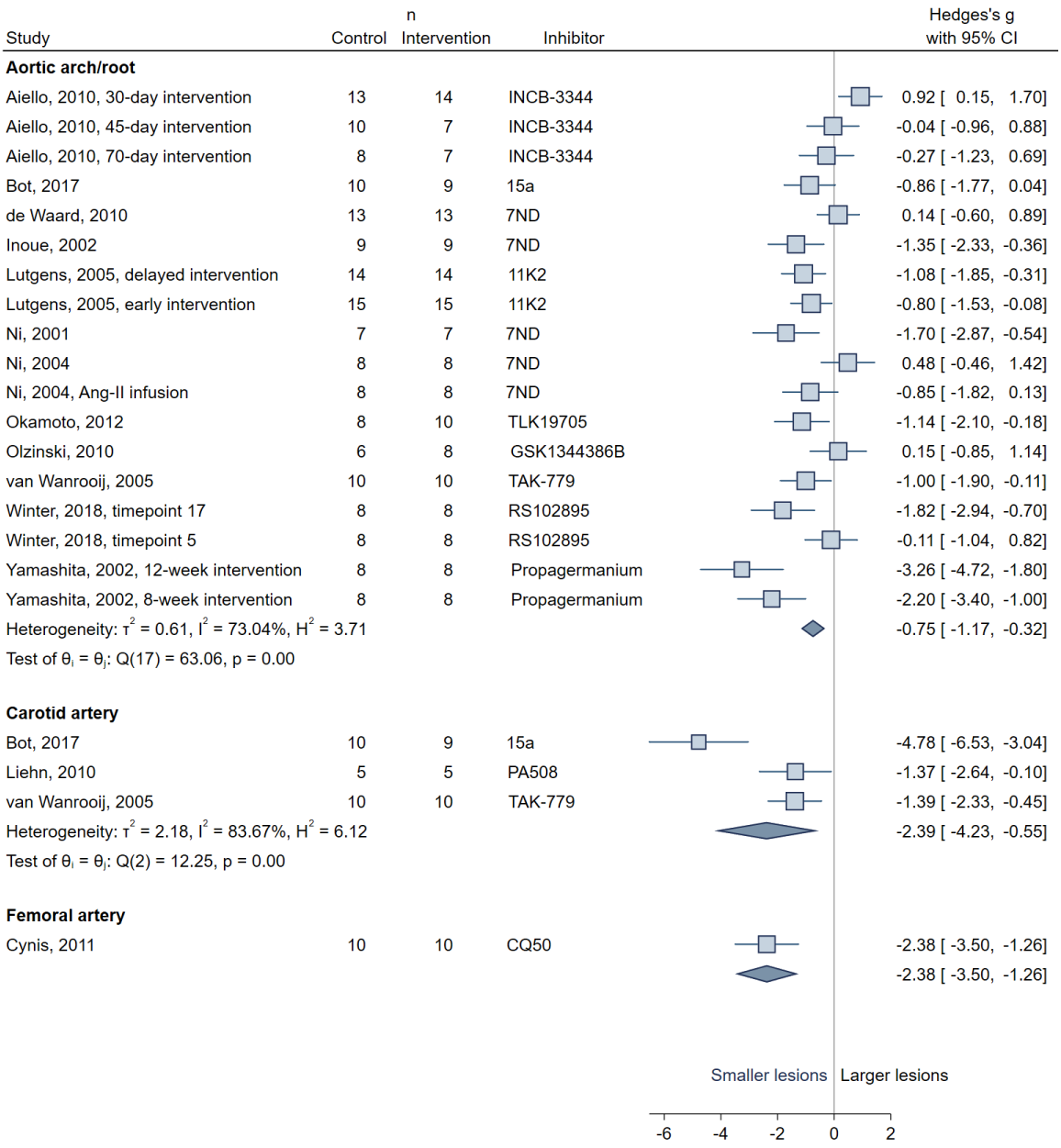

Figure 2. Forest plot of the effects of CCL2/CCR2 inhibition versus control (Hedges' g) on atherosclerotic lesion size in the aortic arch and root, the carotid, and the femoral artery. Shown are the standardized mean differences, calculated as Hedges' *g*, with their respective 95% confidence intervals per study. Plot squares are weighted for study size and correspond to individual effects, whereas plot whiskers correspond to the 95% confidence intervals. Diamonds indicate the pooled effects for each vascular bed. τ^2, I^2 and H^2 as indicators of group heterogeneity as well as test of group homogeneity $\theta_i - \theta_j$ are displayed for the groups containing more than one study.

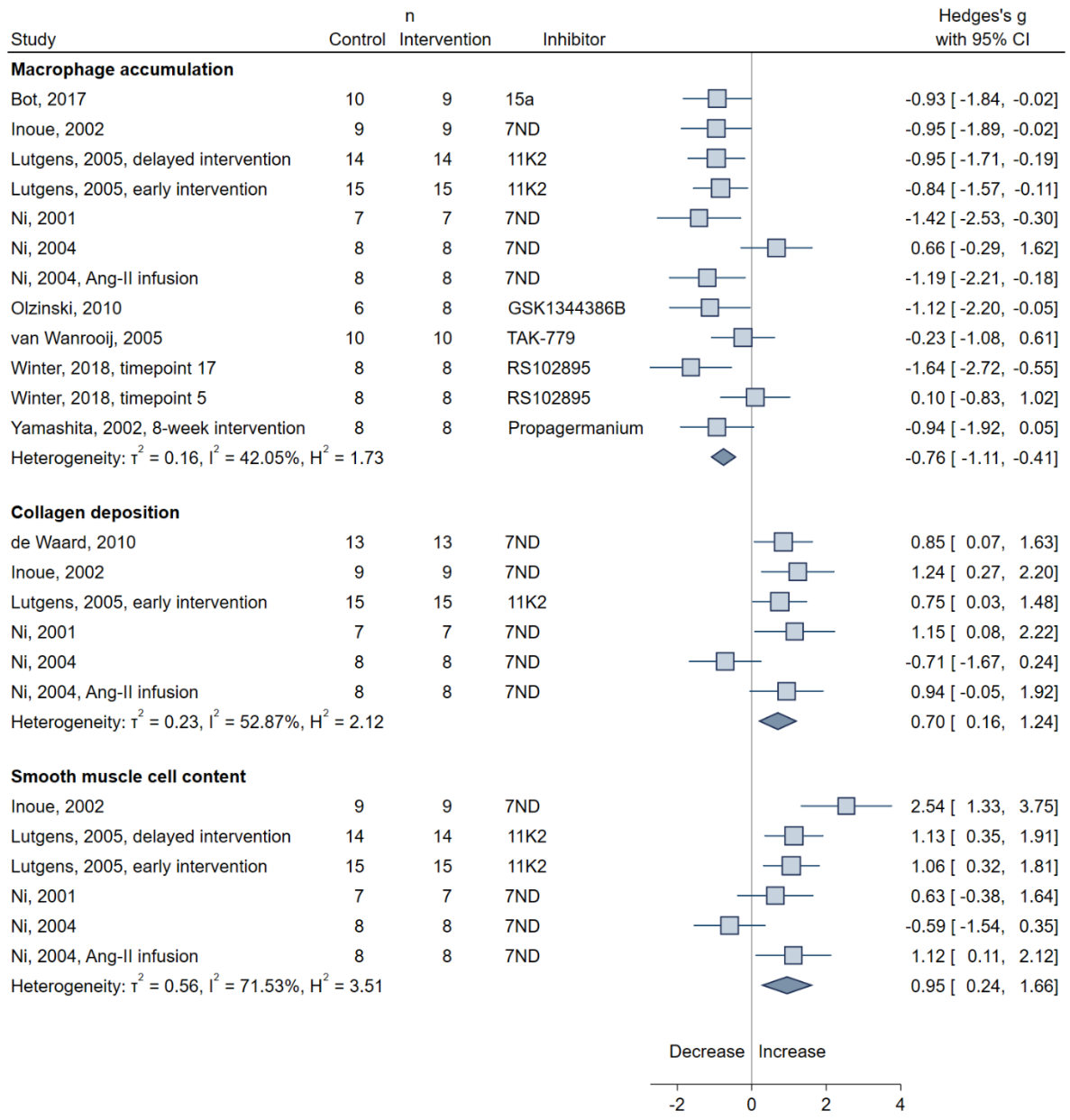

Figure 3. Forest plot of the effects of CCL2/CCR2 inhibition versus control (Hedges' g) on macrophage accumulation, collagen deposition, and smooth muscle content in plaques of the aortic arch or root. Shown are the standardized mean differences, calculated as Hedges' *g*, with their respective 95% confidence intervals per study. Plot squares are weighted for study size and correspond to individual effects, whereas plot whiskers correspond to the 95% confidence intervals. Diamonds indicate the pooled effects for each outcome. τ^2, I^2 and H^2 as indicators of group heterogeneity are displayed for the groups containing more than one study.

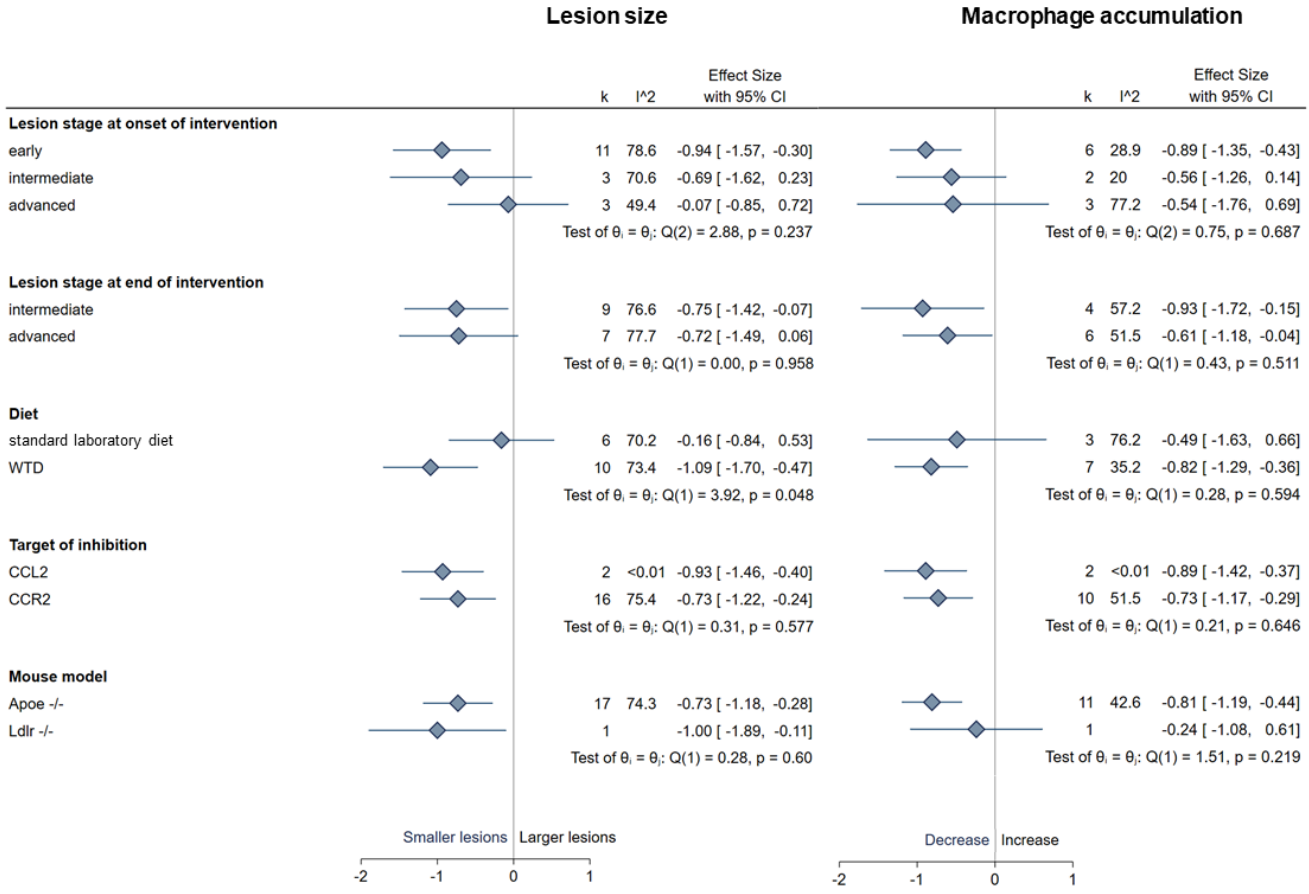

Figure 4. Subgroup analyses regarding the effects of CCL2/CCR2 inhibition versus control (Hedges' g) on aortic plaque burden and macrophage accumulation by various study characteristics. Shown are the pooled standardized mean differences, calculated as Hedges' *g*, with their respective 95% confidence intervals for each subgroup. Diamonds correspond to pooled effects per subgroup, whereas whiskers correspond to the 95% confidence intervals. Number of study arms (*k*) and heterogeneity measures (I^2) per subgroup are displayed. The Cochran's Q test and its p-value are provided as measures of between-subgroup differences. WTD = western-type diet.

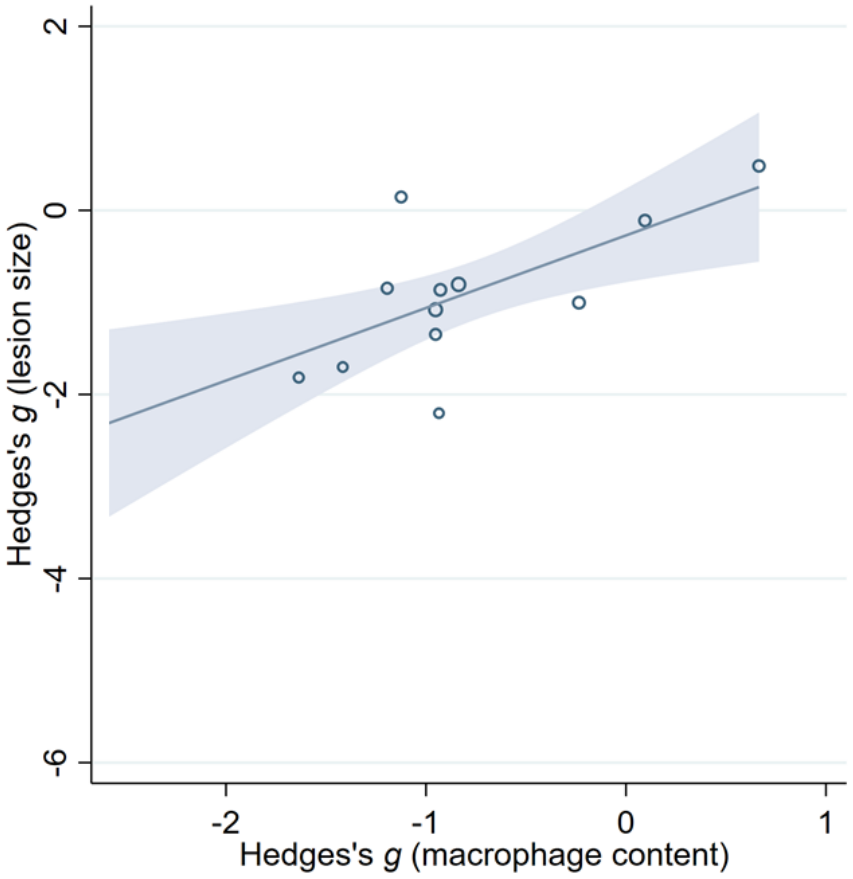

Figure 5. Meta-regression analysis of the effects of CCL2/CCR2 inhibition versus control (Hedges' g) on macrophage accumulation on the effects of the intervention on atherosclerotic lesion size in the aortic arch and root. Data points indicate individual studies around the regression line with its 95% confidence interval (shaded area).